

Induction of Monooxygenation in Rainbow Trout by Polybrominated Biphenyls: A Comparative Study

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Two commercial polychlorinated biphenyl mixtures (Aroclor 1254 and Aroclor 1242) and one polybrominated biphenyl mixture (FireMaster BP-6) were examined for their abilities to induce hepatic microsomal monooxygenation in rainbow trout (*Salmo gairdneri*).

Pretreatment of rainbow trout with Aroclors 1254 and 1242 (150 mg/kg IP) resulted in an approximate 10-fold induction of arylhydrocarbon (benzo[a]pyrene) hydroxylation, ethoxycoumarin-O-deethylation and ethoxyresorufin-O-deethylation within 7 days after injection. These enzyme activities remained elevated above control values for at least 2-3 weeks.

Administration of FireMaster BP-6 (150 mg/kg IP) also resulted in an induction of several monooxygenase activities. Arylhydrocarbon (benzo[a]pyrene) hydroxylation, ethoxycoumarin-O-deethylation and ethoxyresorufin-O-deethylation were increased by 6-, 3-, and 25-fold, respectively. Only the latter two activities remained elevated two weeks post-injection.

Ethylmorphine-N-demethylation was unaffected by the polyhalogenated biphenyls.

Significant increases in P-450 hemoprotein were not observed after pretreatment with any of the polyhalogenated biphenyls studied.

Introduction

The polyhalogenated biphenyls are well recognized as toxic environmental contaminants (1-6), and the polychlorinated biphenyls (PCBs) have been extensively studied as inducers of hepatic microsomal monooxygenation in several species (7-12); however only recently have reports appeared indicating the inducing potential of the polybrominated biphenyls (PBBs) (13-16). These studies have suggested that the PCBs and PBBs are agents which exhibit the induction properties of both the barbiturate and the polycyclic aromatic hydrocarbon classes of inducers. Classically

phenobarbital is a representative member of a class of agents which induce cytochrome P-450, while 3-methylcholanthrene and other polycyclic aromatic hydrocarbons induce cytochrome P₁-450 (P-448).†

Aquatic animals hold an important position in the food chain. Fish are capable of accumulating lipid soluble compounds from their environment leading to large bioconcentration factors (18).

Both PBBs and PCBs have been shown to be accumulated in fish tissues (19, 20), and 2,5,2',5'-tetrachlorobiphenyl was shown to have a half-life of elimination from rainbow trout of 2.66 years (21). Because of the resistance of PBBs and PCBs to metabolic degradation and hence their long half-lives it was of interest to study their induction properties in fish.

Despite initial suggestions to the contrary, it is now recognized that fish possess drug metabolism mechanisms which are similar to those of mammals (22-26). Furthermore, induction of monooxygenation reactions by environmental pollutants, such as the polycyclic aromatic hydrocarbons (27-29) and PCBs (30, 31) have been reported. It has been proposed that field measurements of hepatic arylhy-

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† Although we realize this terminology is an over simplification, we shall use the name cytochrome P-450 for the monooxygenase hemoprotein(s) induced by the phenobarbital type of agents and cytochrome P₁-450 for those induced by the polycyclic aromatic hydrocarbon type of agents. To indicate a nonspecified cytochrome which when reduced and complexed with CO exhibits an absorption maximum around 450 nm, we shall use the term hemoprotein P-450 (17).

drocarbon hydroxylase in fish species may be used as an indicator of aquatic pollution due to petroleum (32).

Although several similarities between mammalian and fish microsomal hemoprotein P-450-mediated monooxygenation exist, a fundamental difference in induction mechanisms has been observed. Although polycyclic hydrocarbons are capable of inducing monooxygenation; inducers of the barbiturate class (e.g. phenobarbital, phenylbutazone, and DDT) are unable to stimulate monooxygenase activity in several species of fish (23, 33, 34).

As previously described, PCBs and PBBs produce a "mixed" type of induction in mammals. Hence, since the mechanism for phenobarbital type induction appears to be absent in fish, this species may be a suitable animal model for study of the induction of P₁-450-like enzymes by polyhalogenated biphenyls.

Materials and Methods

Rainbow trout (*Salmo gairdneri*), weighing 70–100 g, were obtained from Kettle Moraine Springs Trout Hatchery (Adell, Wisconsin), and were held in flowing, charcoal filtered water 12°C for a minimum of two weeks prior to use. A 12-hr (6 AM–6 PM) light cycle was operated.

NADP, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and 7-hydroxycoumarin (umbelliferone) were obtained from Sigma Chemical Company (St. Louis, Missouri). 7-Ethoxycoumarin was synthesized by a published method (35). Ethoxyresorufin was most kindly synthesized by Dr. S. Challend, Wellcome Research Labs, Beckenham, Kent, England, from resorufin obtained from Eastman Kodak, Ltd. Aroclors 1254 and 1242 were generous gifts from Monsanto Chemical Company while metyrapone (Metopirone) was kindly donated by the Ciba-Geigy Corporation (Summit, New Jersey). Dr. D. Rickert (Chemical Industry Institute of Toxicology, Raleigh, North Carolina) generously donated FireMaster BP-6. α -Naphthoflavone and β -naphthoflavone were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). All other reagents were of the highest grade commercially available.

Pretreatment of Fish and Preparation of Microsomes

β -Naphthoflavone, Aroclor 1254, Aroclor 1242, and FireMaster BP-6 were administered to trout by intraperitoneal injection as solutions in corn oil (1 ml/kg). Doses of these compounds varied from 0 to

275 mg/kg. Control fish received equivalent volumes of corn oil alone. After injection fish were maintained in 50-liter tanks (six to eight fish per tank) until sacrifice. Careful injections resulted in no observable leakage of compounds from the injection site.

Fish were sacrificed by a blow to the head, and the gall bladders were carefully removed. The livers were excised and rinsed in ice-cold 0.154M KCl to remove adhering hemoglobin. The livers were minced and washed three times with 0.154M KCl. After washing, the minced livers were homogenized in 4 volumes of 0.25M sucrose by using a motor-driven Potter-Elvehjem-type glass-Teflon homogenizer. The liver homogenates were centrifuged at 8500 g (r_{av} = 8.3 cm) for 20 min by using a Sorval type 24 rotor in a Sorval RC-5 Superspeed centrifuge. The resultant supernatant was decanted and centrifuged at 165,000g (r_{av} = 5.7 cm) for 60 min by using a Beckman type 65 rotor in a Beckman L6-65 ultracentrifuge. The microsomal pellet obtained was resuspended in 0.154M KCl and recentrifuged at 165,000g for 60 min. The washed pellet was suspended in 0.25M sucrose to a concentration equivalent to 1 g wet weight of liver/ml of suspension.

All operations were performed at 0–4°C, and the microsomes were utilized on the day of preparation.

Microsomal Enzyme Assays

Arylhydrocarbon (benzo[a]pyrene) hydroxylation was measured by the method of Hansen and Fouts (36) as modified by Statham et al. (37). Ethoxyresorufin, ethoxycoumarin, and ethylmorphine dealkylations were determined according to published procedures (35, 38, 39).

Temperature optima for these monooxygenase assays were 25°C for arylhydrocarbon hydroxylase and ethylmorphine-N-demethylase and 29–30°C for ethoxycoumarin- and ethoxyresorufin-O-deethylases.

Microsomal protein was measured by the procedure of Ross and Schatz (40) by using crystalline bovine serum albumin standards.

Spectral Studies

Cytochrome P-450 was estimated by the difference in absorbance between the CO complex of Na₂S₂O₄ reduced microsomes and oxidized microsomes by using an extinction coefficient of 100 mM⁻¹·cm⁻¹ between 450 and 510 nm (41). Ethylisocyanide-reduced difference spectra were obtained by the method of Imai and Sato (42). All spectra were obtained at room temperature with a Cary-Varian 219 spectrophotometer.

Results

Pretreatment of rainbow trout with the polyhalogenated biphenyls (FireMaster BP-6, Aroclor 1242, or Aroclor 1254) had no effect on the liver/body ratios or yield of microsomal protein per unit wet weight of liver (Table 1). However, PBBs and PCBs were able to induce the activity of certain monooxygenase reactions.

Dose-response studies of the induction process indicated maximal induction of PBBs and PCBs at about 250 mg/kg (Figs. 1 and 2). Aroclor 1242 maximally induced arylhydrocarbon (benzo[a]pyrene) hydroxylase (AHH) activity by approximately 10-fold, while PBBs resulted in only a 4-fold increase. Ethoxyresorufin-*O*-deethylase was maximally stimulated by approximately 30- and 20-fold by PBBs and Aroclor 1242, respectively.

Table 1. Liver/body weight ratios and yield of microsomal protein.

Treatment	Dose, mg/kg	Liver/body ratio, % ^a	Microsomal protein, mg/g liver ^a
Corn oil		0.96 ± 0.05	24.5 ± 0.8
FireMaster BP-6	150	1.07 ± 0.04	24.0 ± 0.5
Aroclor 1254	150	0.92 ± 0.03	25.0 ± 1.6
Aroclor 1242	150	0.96 ± 0.06	23.6 ± 2.7

^a Values are means ± SE (n = 6).

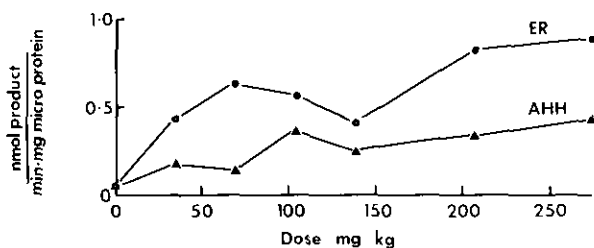


FIGURE 1. Dose-response relationship for Aroclor 1242: (●) ethoxyresorufin-*O*-deethylation; (▲) arylhydrocarbon hydroxylase. Each point represents values from microsomes obtained from the pooled livers of six fish. Values were determined 4 days after injection.

Studies of the time course of induction for Aroclors 1242 and 1254 at a dose of 150 mg/kg indicated that maximal stimulation of monooxygenation was attained after 4 days and 7 days, respectively (Figs. 3 and 4). A single intraperitoneal injection of Aroclor 1242 elevated AHH, ethoxycoumarin-*O*-deethylase, and ethoxyresorufin-*O*-deethylase by about 10-fold at 4 days post-injection. These enzyme activities remained elevated for at least 15 days after treatment of the fish (Fig. 3). Figure 4 shows a similar 10-fold induction of monooxygenase activity by Aroclor 1254. Stimulation of monooxygenation was still apparent 21 days after treatment.

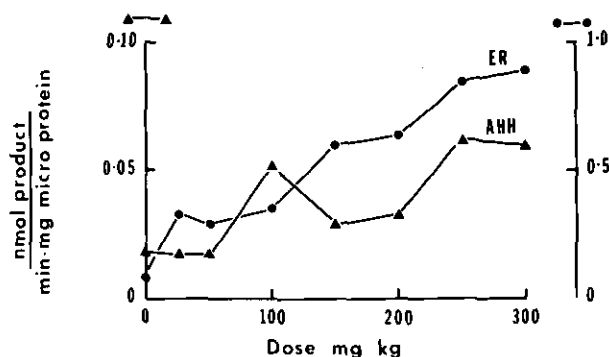


FIGURE 2. Dose-response relationship for FireMaster BP-6: (●) ethoxyresorufin-*O*-deethylation; (▲) arylhydrocarbon hydroxylase. Each point represents values from microsomes obtained from the pooled livers of six fish. Values were determined 4 days after injection.

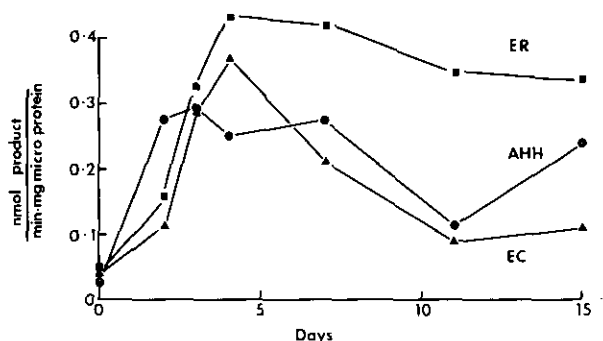


FIGURE 3. Time course of induction by Aroclor 1242: (■) ethoxyresorufin-*O*-deethylation; (▲) ethoxycoumarin-*O*-deethylation; (●) arylhydrocarbon hydroxylase. Each point represents values from microsomes obtained from the pooled livers of six fish. Aroclor 1242 was administered IP in corn oil at a dose of 150 mg/kg.

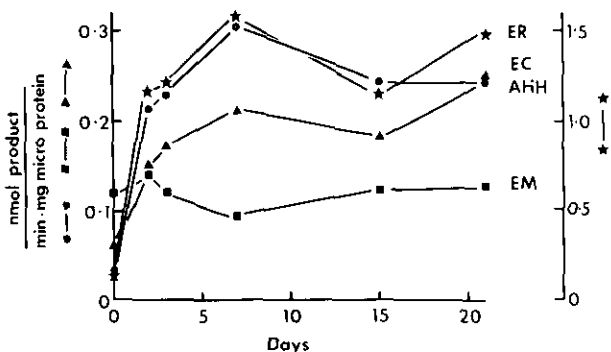


FIGURE 4. Time course for induction by Aroclor 1254: (●) ethoxyresorufin-*O*-deethylation; (▲) ethoxycoumarin-*O*-deethylation; (●) arylhydrocarbon hydroxylase; (■) ethylmorphine-*N*-demethylase. Each point represents values from microsomes obtained from the livers of six fish. Aroclor 1254 was administered IP in corn oil at a dose of 150 mg/kg.

Ethylmorphine-*N*-demethylation was unaffected by pretreatment of fish with Aroclors 1254 or 1242. This is illustrated for Aroclor 1254 in Figure 4.

After injection of rainbow trout with FireMaster BP-6 no increases in monooxygenase activities were seen until 3 days after treatment (Fig. 5). However, at this time AHH, ethoxyresorufin- and ethoxycoumarin-*O*-deethylations were elevated. AHH declined rapidly to reach control levels about 7 days after treatment of the fish, while the latter two activities remained elevated for at least 2 weeks.

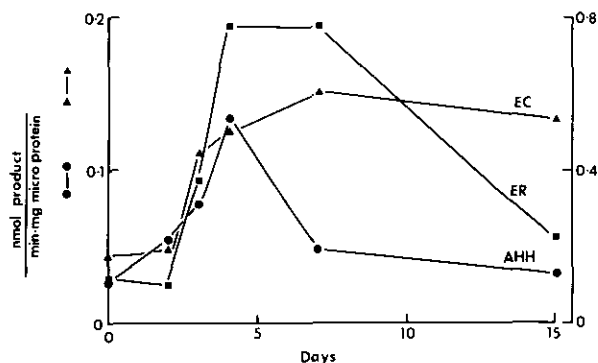


FIGURE 5. Time course for induction by FireMaster BP-6: (■) ethoxyresorufin-*O*-deethylation; (▲) ethoxycoumarin-*O*-deethylation; (●) arylhydrocarbon hydroxylase. Each point represents values from microsomes obtained from the pooled livers of six fish. FireMaster BP-6 was administered IP at a dose of 150 mg/kg.

In common with the PBCs, PBBs also failed to increase ethylmorphine-*N*-demethylation above control values.

The stimulatory effect of PCBs and PBBs upon monooxygenations appears to be a true induction, since inclusion of these compounds in the *in vitro* assays had no effect upon the observed enzymatic activities.

Hemoprotein P-450 concentrations in rainbow trout hepatic microsomes were determined 4 days after pretreatment with various inducing agents. Small increases (10–20%) in hemoprotein P-450 content were observed after treatment of fish with PCBs or PBBs, but these increases were not statistically significant (Table 2). However, β -naphthoflavone pretreatment of fish resulted in a significant 40% increase in the level of P-450 hemoprotein.

It is noteworthy that the λ_{max} for the carbon monoxide complex of reduced cytochrome P-450 was at 449 nm in all preparations examined (Fig. 6).

The interaction of ethylisocyanide with $\text{Na}_2\text{S}_2\text{O}_4$ -reduced cytochrome P-450 resulted in optical difference spectra with absorption maximum at 432–433 nm and 453–454 nm in all microsomal prepara-

tions examined. A small increase in the 455/430 peak ratio was observed after pretreatment with PBBs, PCBs, or β -naphthoflavone (Table 2).

Table 2. Cytochrome P-450 content and 455/430 peak ratios of EtNC spectra.

Treatment	Dose, mg/kg	P-450, nmole/mg protein ^a	EtNC spectra 455/430 ratio ^a
Corn oil		0.118 \pm 0.003	0.28 \pm 0.01
Phenobarbitone	65	0.111 \pm 0.006	0.32 \pm 0.04
FireMaster BP-6	150	0.132 \pm 0.014	0.37 \pm 0.05
Aroclor 1254	150	0.143 \pm 0.020	0.35 \pm 0.05
Aroclor 1242	150	0.137 \pm 0.001	0.39 \pm 0.01
β -Naphthoflavone	100	0.165 \pm 0.005 ^b	0.44 \pm 0.10

^a Values are means \pm SE.

^b Significantly different from corn oil control group, $p < 0.05$ $n = 3$, each determination used pooled microsomes from the livers of 2 fish.

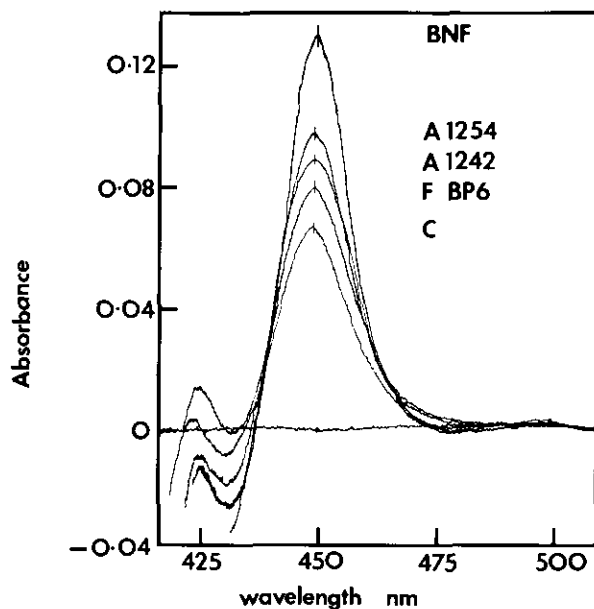


FIGURE 6. Hemoprotein P-450 difference spectra: (BNF) β -naphthoflavone (100 mg/kg); (A1254) Aroclor 1254 (150 mg/kg); (A1242) Aroclor 1242 (150 mg/kg); (FBP6) FireMaster BP-6 (150 mg/kg); (C) corn oil (1 ml/kg). Microsomes (2–4 mg/ml) were divided between two cuvettes. A baseline of equal light absorbance was obtained and CO was bubbled through both cuvettes. A few mg of $\text{Na}_2\text{S}_2\text{O}_4$ was added to the sample cuvette and the resultant spectrum between 420 and 510 nm recorded.

Discussion

The present study has demonstrated that PCBs and PBBs are capable of stimulating hepatic microsomal monooxygenation reactions in rainbow trout following a single intraperitoneal injection. In general, Aroclor 1254 and Aroclor 1242 appeared to be more potent than FireMaster BP-6; however the latter compound was much more effective at in-

ducing ethoxyresorufin-*O*-deethylation.

Notably Aroclor 1242 treatment resulted in monooxygenase activities, attaining a maximum at about 4 days, while in Aroclor 1254-treated fish maximal stimulation was not observed until 7 days. FireMaster BP-6 increased AHH and ethoxyresorufin-*O*-deethylation by 4 days after injection, however ethoxycoumarin-*O*-deethylation did not reach a maximum until 7 days after injection. This has also been observed in rats treated with FireMaster BP-6 (14).

The inducing properties of the polyhalogenated biphenyls appear to be expressed differently in fish than in rodents. The polyhalogenated biphenyls failed to increase liver/body weight ratios or yield of microsomal protein; furthermore increases in ethylmorphine-*N*-demethylase were not apparent. These responses are typical of polyhalogenated biphenyl induction in rodents (10-15, 43) and are characteristic of phenobarbital-type (cytochrome P-450) induction. PCB and PBB treatment of rainbow trout were found to stimulate AHH, ethoxycoumarin-*O*-deethylation and ethoxyresorufin-*O*-deethylation. AHH and ethoxycoumarin-*O*-deethylation are induced by both phenobarbital and 3-methylcholanthrene in rodents; however ethoxyresorufin-*O*-deethylation is specifically inducible by the polycyclic aromatic hydrocarbon type of inducing agents [cytochrome P₁-450] (38).

Because of the ability of commercial polyhalogenated biphenyls to elicit both phenobarbital-like and 3-methylcholanthrene-like induction, the term 'mixed' inducer has frequently been used. It has been demonstrated that cytochrome P₁-450 or cytochrome P-450 induction by PCBs is determined by the substitution pattern of the halogen atoms (43-45). For example 2,2',4,4',5,5'-hexachlorobiphenyl was found to induce a cytochrome P-450, while 3,4,3',4'-tetrachlorobiphenyl specifically induced cytochrome P-448. More recently (46, 47) it has been suggested that a similar situation exists with the PBBs. These workers have demonstrated that 2,4,5,2',4',5'-hexabromobiphenyl (the major component of FireMaster BP-6) is a cytochrome P-450 inducer (phenobarbital type). By extrapolation from data obtained using the PCBs it is logical to assume that certain PBB isomers may induce cytochrome P₁-450. However, some evidence exists for the hypothesis that the 'mixed' properties of PBB-induced microsomal preparations are not due to the presence of cytochrome P₁-450 (48).

In contrast to the apparent "mixed" patterns of stimulation of monooxygenation in rodents due to polyhalogenated biphenyls, rainbow trout seem incapable of responding to phenobarbital-type (cytochrome P-450) induction.

The large increases (up to 25-30-fold) in monooxygenase activity observed after PCB or PBB treatment of fish cannot be explained in terms of an increase in total hemoprotein P-450 concentrations, since the levels of this (these) enzyme(s) were increased only by about 10-20%. Hence it is possible that a novel enzyme with different substrate specificity is induced by the PBBs and PCBs in rainbow trout. However, no changes in the wavelength of the absorption maximum of the CO complex of ferrocytochrome P-450 were seen, nor were the ratios of the 450 and 430 nm absorption maxima of the ethylisocyanide complex of ferrocytochrome P-450 significantly altered. In rodents, the 455-530 peak ratio increases considerably after treatment with PCBs or PBBs (9, 43, 48).

Further studies utilizing specific inhibitors of cytochrome P₁-450 (α -naphthoflavone) and cytochrome P-450 (metyrapone) demonstrated no distinct differences in the pattern of inhibition of monooxygenation in PCB, PBB or control hepatic microsomes obtained from rainbow trout (Elcombe, unpublished data).

In conclusion, it is apparent that PCBs and PBBs are potent and persistent inducers of certain monooxygenase reactions in the rainbow trout. These compounds, which show a "mixed" type of induction in rodents, only elicit a polycyclic aromatic hydrocarbon type of stimulation in the rainbow trout. However, unlike the mammalian situation, this stimulation does not appear to be accompanied by the synthesis of cytochrome P₁-450.

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